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Photophysiology of the Elongated Internode (*ein*) Mutant of *Brassica rapa*[1]

ein Mutant Lacks a Detectable Phytochrome B-Like Polypeptide

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ABSTRACT

Several phytochrome-controlled processes have been examined in etiolated and light-grown seedlings of a normal genotype and the elongated internode (*ein*/*ein*) mutant of rapid-cycling *Brassica rapa*. Although etiolated *ein* seedlings displayed normal sensitivity to prolonged far-red light with respect to inhibition of hypocotyl elongation, expansion of cotyledons, and synthesis of anthocyanin, they displayed reduced sensitivity to prolonged red light for all three of these deetiolation responses. In contrast to normal seedlings, light-grown *ein* seedlings did not show a growth promotion in response to end-of-day far-red irradiation. Additionally, whereas the first internode of light-grown normal seedlings showed a marked increase in elongation in response to reduced ratio of red to far-red light, *ein* seedlings showed only a small elongation response. When blots of protein extracts from etiolated and light-treated *ein* and normal seedlings were probed with monoclonal antibody to phytochrome A, an immunostaining band at about 120 kD was observed for both extracts. The immunostaining intensity of this band was substantially reduced for extracts of light-treated normal and *ein* seedlings. A mixture of three monoclonal antibodies directed against phytochrome B from *Arabidopsis thaliana* immunostained a band at about 120 kD for extracts of etiolated and light-treated normal seedlings. This band was undetectable in extracts of *ein* seedlings. We propose that *ein* is a photoreceptor mutant that is deficient in a light-stable phytochrome B-like species.

The phytochrome photoreceptor system regulates growth and development throughout the life history of higher plants. Physiological and biochemical analyses of phytochrome and phytochrome responses, in both etiolated and light-grown plants, have led to the concept of two phytochrome pools—a light-labile pool, which accumulates in etiolated tissues, and a light-stable pool, which is unaffected by light (7, 21). More recently, it has been directly established that higher plants contain multiple, discrete species of phytochrome, the products of a small family of divergent genes (20). In *Arabidopsis thaliana*, at least five phytochrome-related sequences have been identified by genomic DNA analysis, and three full-length cDNA clones, *phyA*, *phyB*, and *phyC*, corresponding to three of these genes, have been isolated (16, 20). Phytochrome A, encoded by the *phyA* gene, is a light-labile species that accumulates in etiolated plant tissues. Phytochromes B and C are light-stable species (22).

The existence of multiple phytochrome species raises the question of what roles the individual phytochromes might play within the spectrum of plant photomorphogenesis. The identification and analysis of strains carrying mutations in the various phytochrome structural genes will undoubtedly assist in addressing this question. Several candidate phytochrome mutations in a number of plant species have been reported (10). At present, only three of these, the cucumber *lh*, *Arabidopsis hy3*, and sorghum *ma3*^{R} are considered to be good candidates for phytochrome apoprotein mutations (17). The cucumber *lh* and *Arabidopsis hy3* mutants display a light-dependent pleiotropic phenotype that is similar to the low R/FR[2] ratio-induced shade avoidance syndrome. Thus, these mutants display an elongated growth habit, are defective in end-of-day FR elongation growth responses, flower early compared with their isogenic wild types, and respond poorly to reduced R/FR ratio (10, 14, 24). Both of these mutants have been shown to be wholly or partially deficient in a phytochrome polypeptide that can be detected with monoclonal antibodies directed against phytochrome B (13, 14, 22). For *hy3*, it has also been established that, compared with wild-type seedlings, hybridizable *phyB* mRNA levels are reduced (22).

The sorghum *ma3*^{R} mutation, which is photoperiod insensitive and flowers early, results in increased levels of gibberellins and displays an elongated phenotype (6). Some features of the sorghum *ma3*^{R} phenotype are similar to the low R/FR ratio-induced shade avoidance syndrome. Recently, this mu-

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[2] Abbreviations: R, red light; FR, far-red light; R/FR ratio, the photon fluence rate ratio of red to far-red light in 10-nm bandwidths centered on 660 nm and 730 nm; W(f/i), white light comprising a mixture of white fluorescent and incandescent light.

tant was shown to be deficient in a low-abundance polypeptide that is immunostainable with both a monoclonal antibody raised against pea phytochrome and directed against a highly conserved epitope (6) and a monoclonal antibody raised against phytochrome from light-grown oat shoots.(5).

The elongated internode, *ein*, mutant of *Brassica rapa* (syn. *campestris*) arose fairly recently as the result of a naturally occurring mutation in a rapid-cycling line of *B. rapa*. The *ein* mutation results in elevated levels of gibberellins (18), and various aspects of growth and development have previously been compared in *ein* versus other genotypes (19, 25). The *ein* mutation leads to a pleiotropic phenotype that also resembles the low R/FR ratio-induced shade avoidance syndrome. Here, we report that the *ein* mutation leads to a light-conditional, constitutive shade avoidance phenotype and that *ein* seedlings lack a phytochrome polypeptide that is immunodetectable by monoclonal antibodies directed against phytochrome B.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of a normal, rapid-cycling line of *Brassica rapa* (syn. *campestris*) (designation: CrGC 1–1) and the elongated internode, *ein/ein* mutant (designation: CrGC 1–11) were obtained from the Crucifer Genetics Cooperative (University of Wisconsin-Madison).

For experiments with etiolated seedlings, seeds were placed on Whatman No. 1 filter paper moistened with BG11 mineral nutrient solution (23) and chilled (4°C) in the dark for 3 d. Seeds were germinated at 20°C in complete darkness for 1 d. For experiments with light-grown seedlings, seeds were germinated as above and seedlings transplanted into a 3:1 mixture of peat compost:horticultural silver sand. Seedlings were deetiolated for the specified times under continuous white fluorescent light provided by Osram L65/80W/30 warm white fluorescent tubes (photon fluence rate, 400–700 nm, 153 μmol $m^{-2}s^{-1}$).

Light Sources

Broad band R at a photon fluence rate (600–700 nm) of 5 μmol $m^{-2}s^{-1}$ was obtained by filtering the output from Thorn E.M.I. (Birmingham, UK). Deluxe natural 40-W fluorescent tubes through a 1-cm deep layer of copper sulfate solution (1.5% w/v) and one layer of red (No. 14) cinemoid (Rank Strand, Isleworth Middlesex, UK). Broad band FR at a photon fluence rate (700–800 nm) of 27 μmol $m^{-2}s^{-1}$ was obtained by filtering the output from water-cooled 100-W incandescent bulbs through black plexiglass (type FRF 700; West Lakes Plastics, Lenni, PA). W(f/i), at a photon fluence rate (400–800 nm) of 110 μmol $m^{-2}s^{-1}$, was provided by a Fi-totron 600 growth cabinet (Fisons Scientific Apparatus, Loughborough, UK).

The R/FR ratio treatment cabinets were the same as those described in detail by Keiller and Smith (9). The high R/FR ratio cabinet (cool-white fluorescent light) provided a photon fluence rate (400–700 nm) of 189 μmol $m^{-2}s^{-1}$ and a R/FR ratio of 7.12. The low R/FR cabinet (cool-white fluorescent light supplemented with FR) provided the same photon fluence rate (400–700 nm) but a R/FR ratio of 0.17. All light measurements were made using a LI 1800/12 spectroradiometer (Li-Cor, Lincoln, NE).

Growth Measurements

Hypocotyl and internode lengths were measured with a ruler. All data represent the mean hypocotyl/internode lengths from between 10 and 50 seedlings, depending upon the experiment, for each treatment. Cotyledon areas were determined by placing excised cotyledons between microscope slides next to a reference square of known area and photographing them. Transparencies were projected, and outlines of the cotyledons and reference square were then traced from the screen onto paper, cut out, and fed through a leaf area meter from which the original cotyledon areas were calculated. Data were obtained from 10 cotyledon pairs for each treatment.

Measurement of Anthocyanin

Anthocyanin extraction and assay were performed exactly as described by Adamse et al. (3). Following extraction in 1% (w/v) HCl in methanol and Folch partitioning, the anthocyanin content of 10 seedlings was recorded as A_{535}. Each extraction was repeated four times to obtain a mean.

Phytochrome Extraction and Immunoblotting

Plant tissue was harvested into liquid nitrogen and stored at −70°C. Extractions from 2- to 3-g batches of tissue were performed as described by Somers et al. (22). Briefly, frozen tissue was homogenized with a volume of extraction buffer equivalent to the fresh weight of tissue (extraction buffer: 50% [v/v] ethylene glycol; 100 mM Tris-HCl, pH 8.5; 150 mM $(NH_4)_2SO_4$; 10 mM EDTA; 60 mM $NaHSO_3$; aprotinin [2 μg mL^{-1}]; leupeptin [1 μg mL^{-1}]; pepstatin [1 μg mL^{-1}]; 2 mM PMSF; and 10 μM iodoacetamide). Following polyethyleneimine precipitation and centrifugation, the supernatant was subjected to $(NH_4)_2SO_4$ precipitation (0.25 g mL^{-1}). Pelleted proteins were resuspended to 5% of the original supernatant volume in half-strength extraction buffer, mixed with an equal volume of gel sample buffer (12), frozen in liquid nitrogen, and stored at −70°C for subsequent gel electrophoresis. Samples containing 25 or 50 μg of protein were resolved on 9% (w/v) SDS-polyacrylamide (12) minigels and electroblotted onto nitrocellulose (Hybond C; Amersham, UK). Protein blots were probed with the following monoclonal antibodies: (a) 073d, specific for phytochrome A; (b) B1, B7, and B8, specific for phytochrome B; (c) C1, C11, and C13, specific for phytochrome C (22). Secondary incubations were carried out with anti-mouse immunoglobulin antibodies conjugated to alkaline phosphatase. Bands were visualized by development of blots in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

RESULTS

Photoresponses of Etiolated *ein* and Normal Plants

Hypocotyl Elongation

One-day-old etiolated seedlings of *ein* and normal plants were grown for 2 d in darkness, broad band R, broad

band FR, or W (f/i), after which hypocotyl lengths were determined.

In etiolated normal seedlings, hypocotyl elongation was substantially inhibited, relative to darkness, in response to growth under broad band R or FR and W(f/i) (Figs. 1a and 2). The hypocotyls of etiolated *ein* seedlings maintained in darkness elongated to a similar extent as those of normal seedlings, suggesting that the elongated phenotype of *ein* seedlings is light conditional. In response to continuous broad band FR, the hypocotyls of etiolated *ein* seedlings were inhibited to almost the same extent as those of normal seedlings. However, hypocotyls of R-treated *ein* seedlings were significantly longer than those of normal seedlings. The long hypocotyl phenotype was also observed for *ein* seedlings grown under W(f/i) (Figs. 1a and 2). Thus, the elongated phenotype of *ein* seedlings appears to be due to a deficiency in the inhibitory action of R, whereas the action of continuous FR is unaffected by the mutation.

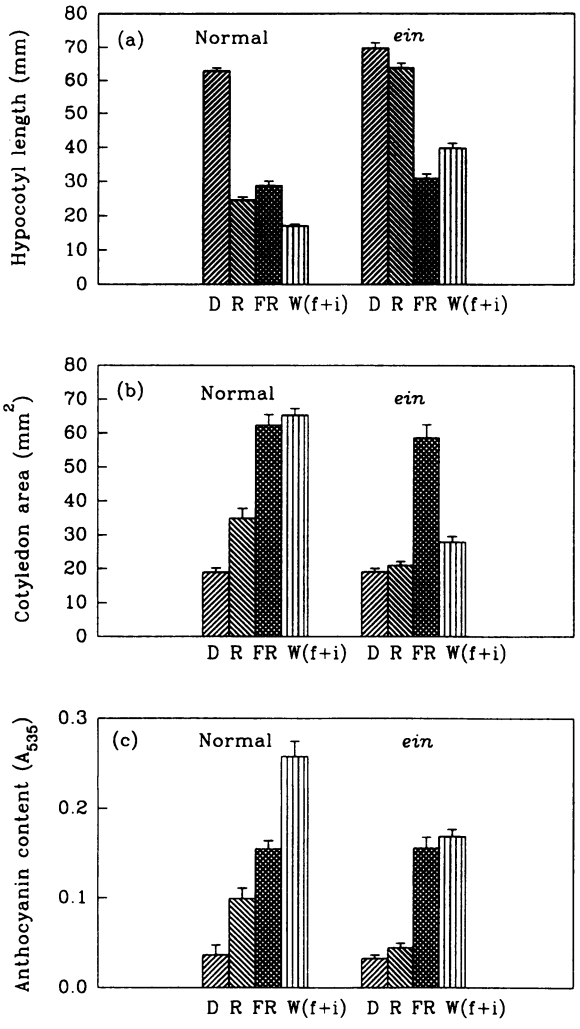

Figure 1. Phytochrome-controlled responses of etiolated normal and *ein* seedlings. One-day-old etiolated seedlings were placed in continuous R, FR, or W(f/i) for 2 d. a, Mean hypocotyl lengths (± SE) of seedlings; b, cotyledon areas (±SE); c, anthocyanin contents, measured as A_{535} (±SE). D, Dark.

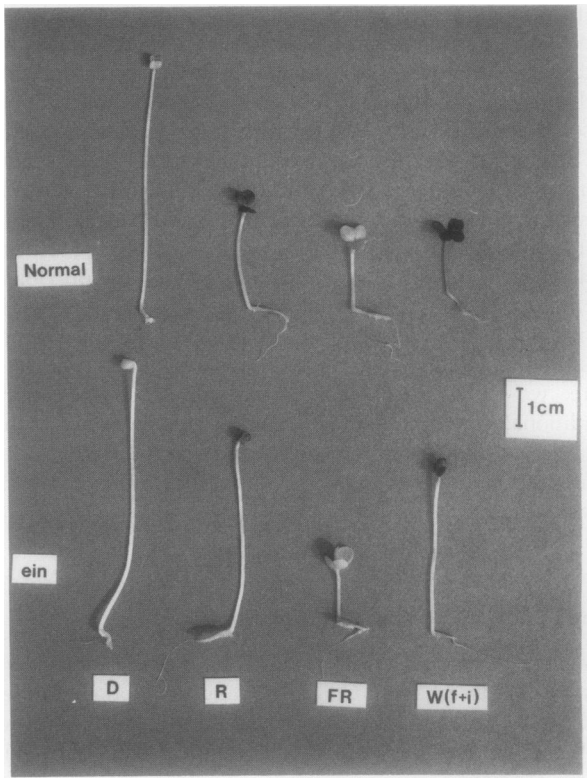

Figure 2. The R-dependent long hypocotyl phenotype of *ein* seedlings. One-day-old normal and *ein* seedlings were maintained in the dark (D) or placed in continuous R, FR, or W(f/i) for 2 d.

Cotyledon Expansion

Cotyledon expansion in etiolated normal seedlings was substantially promoted by exposure to R, FR, and W(f/i) (Fig. 1b). In contrast, although exposure of etiolated *ein* seedlings to continuous FR induced cotyledon expansion to the same extent as it did in normal seedlings, exposure of *ein* seedlings to R or W(f/i) led to significantly reduced cotyledon expansion compared with normal seedlings (Fig. 1b). Thus, as for hypocotyl elongation, *ein* seedlings display a normal response to FR but a reduced response to R and white light.

Anthocyanin Synthesis

Exposure of etiolated normal seedlings to R, FR, or W(f/i) caused significant accumulation of anthocyanin (Fig. 1c). However, although *ein* seedlings showed a normal accumulation of anthocyanin in response to FR, they showed reduced anthocyanin accumulation following exposure to R and W(f/i) (Fig. 1c).

Thus, for three separate phytochrome-regulated responses of deetiolation, *ein* seedlings display a normal response to FR and a selective deficiency in the action of R.

Photoresponses of Light-Grown *ein* and Normal Seedlings

End-of-Day FR Growth Response

Seedlings of *ein* and normal plants were deetiolated for 1 d in continuous white fluorescent light and then grown in 12-h light/12-h dark cycles. At the end of each 12-h day, plants were exposed to FR for 15 min immediately before transfer to darkness. Control plants were transferred to darkness without exposure to FR. Mean hypocotyl lengths for each treatment were measured at the end of each light or dark period.

Normal seedlings showed pronounced elongation growth promotion in the hypocotyl in response to end-of-day FR light treatment (Fig. 3). In contrast, the already elongated hypocotyls of *ein* seedlings showed no detectable elongation growth promotion by end-of-day FR (Fig. 3).

R/FR Ratio Growth Response

Light-grown *ein* and normal seedlings were grown in white fluorescent light to first internode production and were then treated under high or low R/FR ratio. The first internode of normal seedlings showed a typical, pronounced elongation growth promotion in response to supplementary FR (Fig. 4). The first internode of *ein* seedlings showed only a slight elongation growth promotion in low, compared with high, R/FR ratio (Fig. 4).

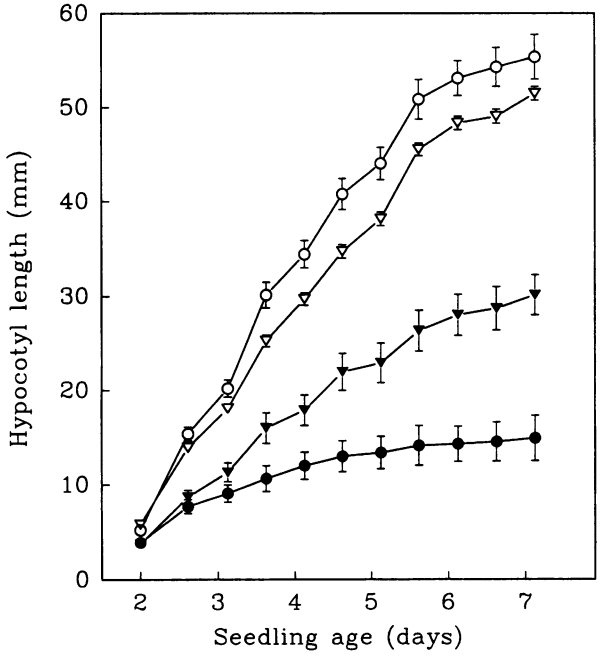

Figure 3. Effect of end-of-day FR treatment on hypocotyl length in light-grown normal and *ein* seedlings. Following deetiolation for 1 d in continuous white fluorescent light, normal (closed symbols) and *ein* (open symbols) seedlings were grown under 12-h light/12-h dark cycles, with (triangles) or without (circles) a 15-min FR treatment immediately before transfer to darkness. Results are means ± SE.

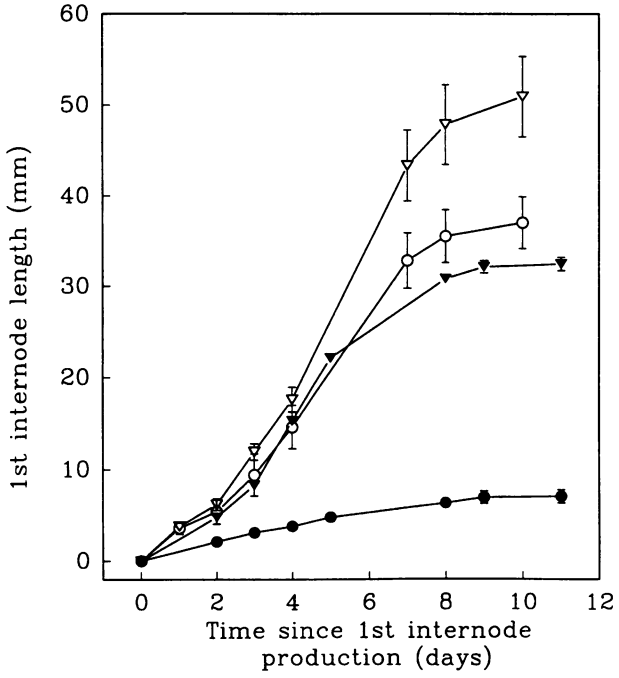

Figure 4. R/FR ratio and first internode elongation in light-grown normal and *ein* seedlings. Normal (closed symbols) and *ein* (open symbols) seedlings were grown to first internode production in white fluorescent light (16-h light/8-h dark cycles) and then either maintained under these high R/FR ratio conditions (circles) or transferred to white fluorescent light supplemented with FR (triangles). Results are means ± SE.

Immunodetection of Phytochromes in Etiolated and Light-Grown *ein* and Normal Seedlings

Protein extracts from etiolated and light-treated *ein* and normal seedlings were resolved by SDS-PAGE, transferred to nitrocellulose, and immunostained with a range of monoclonal antibodies directed against different phytochrome molecular species. The monoclonal antibody 073d, originally raised to purified etiolated oat phytochrome and shown to selectively recognize phytochrome A of *Arabidopsis* (22), immunostained a single polypeptide of about 120 kD in extracts of both etiolated *ein* and normal seedlings (Fig. 5A). The intensity of staining of this polypeptide by 073d was greatly reduced in extracts of light-treated *ein* and normal seedlings (Fig. 5A).

Immunostaining of similar blots using monoclonal antibodies B1, B7, and B8, raised to and shown to be specific for phytochrome B of *Arabidopsis* (22), revealed a single polypeptide band at about 120 kD for extracts of etiolated normal seedlings (Fig. 5B). This polypeptide was also immunodetectable at only slightly reduced levels in blots of extracts of light-treated normal seedlings (Fig. 5B). In contrast, these monoclonal antibodies yielded no detectable staining for extracts of either etiolated or light-treated *ein* seedlings (Fig. 5B). Even following prolonged immunodevelopment, these monoclonal antibodies failed to detect any band in extracts of *ein* seedlings.

When similar blots were immunostained using monoclonal

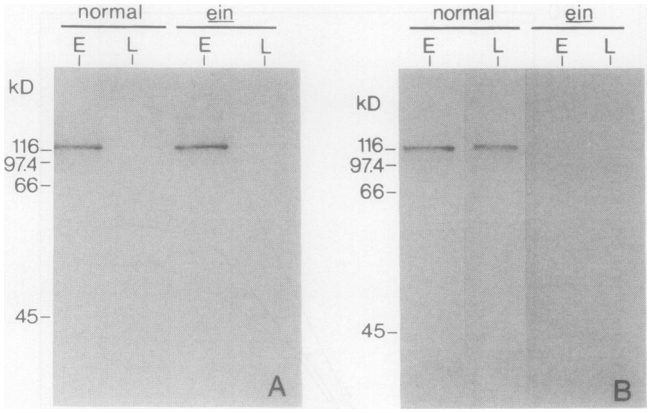

Figure 5. Immunoblots of crude protein extracts of etiolated and light-treated normal and *ein* seedlings following electrophoresis on 9% (w/v) SDS-polyacrylamide gels. A, Immunoblots of extracts, containing 25 μg of protein, from etiolated (E) and light-treated (L) normal and *ein* seedlings were probed with the phytochrome A-selective monoclonal antibody 073d. B, Immunoblots of extracts, containing 25 μg of protein, from etiolated (E) and light-treated (L) normal and *ein* seedlings were probed with the phytochrome B-selective monoclonal antibodies B1, B7, and B8. Light-treated seedlings were irradiated with continuous R for 24 h.

antibodies C1, C11, and C13, shown to be specific for phytochrome C of *Arabidopsis* (22), no immunostaining bands were detected for extracts of either etiolated or light-treated *ein* or normal seedlings (data not shown).

DISCUSSION

It has recently been established that the *ein* mutant of *B. rapa* has elevated endogenous levels of gibberellins (18). A role for phytochrome in the regulation of gibberellin metabolism has been frequently reported (6, 15). This, together with the similarity between the phenotype of the *ein* mutant and the appearance of seedlings following growth under low R/FR ratio, prompted us to examine the phytochrome status and photophysiology of this mutant. We propose that *ein* is a phytochrome-related mutant.

Results of immunoblot analyses using monoclonal antibodies, shown to be selective for different phytochrome molecular species from *Arabidopsis*, provide a good indication that *ein* seedlings possess substantially reduced levels of a polypeptide that can be stained by antibodies against phytochrome B. A monoclonal antibody shown to be selective for phytochrome A stains a light-labile polypeptide of approximately 120 kD from extracts of both *ein* and normal seedlings. We suggest that this is the phytochrome A homolog of *B. rapa*. The approximate 120-kD polypeptide, detectable in extracts of etiolated and light-treated normal seedlings by immunostaining with a mixture of three monoclonal antibodies directed against phytochrome B, is proposed to be a type II phytochrome homolog of *B. rapa*. In the absence of amino acid sequence data, it cannot be stated with certainty that this is the *B. rapa* phytochrome B homolog, although it is probable. The polypeptide stained by these antibodies is not detectable in extracts of either etiolated or light-treated *ein* seedlings, indicating a substantially reduced abundance. This is taken as good evidence that *ein* is a photoreceptor mutant, although the reason for the deficiency in this phytochrome species is not yet known.

When immunoblots of extracts from etiolated and light-treated normal and *ein* seedlings were probed with a mixture of monoclonal antibodies directed against *Arabidopsis* phytochrome C, no detectable staining was observed. The reason for this lack of staining is not known, but one possibility is that the epitopes recognized by the monoclonal antibodies, which are present on *Arabidopsis* phytochrome C, are not conserved in the *B. rapa* homolog of phytochrome C. Alternatively, because *Arabidopsis* phytochrome C is known to be present at only 10% of the level of phytochrome B (22), it is possible that the *B. rapa* homolog is below the level of detection under the present assay conditions. This uncertainty, together with the current lack of knowledge about the phytochrome family of *B. rapa*, means that it is not yet possible to be certain that the *ein* mutant is deficient in only one phytochrome species.

The photophysiology of *ein* is consistent with selective loss of activity of a light-stable phytochrome species, and in several respects the photoresponses of *ein* resemble those of other phytochrome-deficient mutants. Thus, during deetiolation under prolonged R, *ein* seedlings display reduced inhibition of hypocotyl elongation compared with normal seedlings, whereas *ein* seedlings show normal inhibition of hypocotyl elongation under prolonged FR. Growth inhibition by prolonged FR probably reflects the operation of the high irradiance response (4), a response mode thought to be mediated by light-labile phytochrome A (21). Inhibition of hypocotyl elongation by prolonged R is thought to be, at least partly, mediated by a light-stable phytochrome species (4) and has been shown to be reduced in the phytochrome B-deficient *Arabidopsis hy3* mutant (11) and the phytochrome B-deficient cucumber *lh* mutant (2). Thus, for hypocotyl growth, *ein* seedlings appear to display normal phytochrome A action but reduced action of a B-like phytochrome.

When grown in darkness, *ein* seedlings elongate slightly more than normal seedlings, suggesting that the *ein* phenotype is not absolutely light conditional. This growth difference, which is not a consequence of green safelight exposure (cf. ref. 2), may reflect some residual influence of the seed. The possibility that this residual seed effect may be attributable to seed Pfr was tested by irradiating imbibed seeds with FR to remove any stable Pfr; however, this did not eliminate the growth difference. We observed that *ein* seeds are about 30% larger than normal seeds. It is possible that different growth characteristics of the parent *ein* or normal plants affect seed development differently and that this results in differences in subsequent seedling growth.

The retention of a normal response to prolonged FR, but loss of response to R, in *ein* is also seen for two other phytochrome-regulated aspects of deetiolation, namely, anthocyanin synthesis and cotyledon expansion. Anthocyanin synthesis is a classical phytochrome-controlled response of etiolated seedlings of many species, including *B. rapa* (8). The reduced accumulation of anthocyanin in *ein* seedlings in response to R confirms the pleiotropic nature of the *ein* mutant phenotype and supports the notion that *ein* is a

photomorphogenetic mutant. The sorghum *ma3*^{R} mutant, a putative phytochrome mutant, has also been reported to display aberrant phytochrome-controlled anthocyanin accumulation (6). Cotyledon expansion is phytochrome regulated in many etiolated seedlings, and light-dependent cotyledon expansion has been shown to be reduced in the phytochrome B-deficient cucumber *lh* mutant (2).

The growth responses of light-grown *ein* seedlings are also consistent with reduced action of a light-stable phytochrome species. Light-grown *ein* seedlings do not display a growth promotion by end-of-day FR, whereas this response is observed in normal seedlings. End-of-day FR growth promotion, which has become a standard "assay" for the action of light-stable phytochrome (10), is also not observed in the *Arabidopsis hy3* and cucumber *lh* mutants (1, 14). Additionally, the first internode of *ein* seedlings displays only a small growth promotion in response to seedling growth under low R/FR. The *Arabidopsis hy3* and cucumber *lh* mutants have also been reported to show a diminished response to low R/FR ratio (24).

In conclusion, the *ein* mutant of *B. rapa,* originally characterized as a gibberellin overproducer, is proposed to be a phytochrome-related mutant. Evidence from both immunochemical and photophysiological studies is consistent with the view that *ein* has reduced amounts of a light-stable phytochrome species. The phytochrome species lacking in the *ein* mutant is probably phytochrome B, although confirmation of this and identification of the reason for the deficiency await further study. In light of the fact that rapid-cycling brassicas are ideal plants for photomorphogenetic studies, further characterization of this mutant is likely to assist in determining the roles played by different phytochromes. Furthermore, the physiology of this mutant in which a phytochrome deficiency alters gibberellin status supports the long-proposed linkage between phytochrome and gibberellins.

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